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# A hybrid lineage derived from hybridization of *Carassius cuvieri* and *Carassius auratus* red var. and a new type of improved fish obtained by back-crossing

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ABSTRACT

Hybridization is an important technique and is widely used in fish genetic breeding; this technique prevents variety degeneration and improves variety. In this research, we obtained a hybrid lineage (WR-$F_1$-$F_4$) by interspecific hybridization derived from female white crucian carp (*Carassius cuvieri*, WCC) and male red crucian carp (*Carassius auratus* red var., RCC), and a new type of improved fish (WR-II) was obtained by back-crossing between the female hybrid (WR-$F_1$) and the male WCC. We investigated the morphological traits, chromosomal numbers, DNA content, gonadal development, sperm shape and 5S rDNA in the hybrid lineage and WR-II. The results showed that all the assessed morphological traits were not significantly different between the progenies of the hybrid lineage and their original parents, but the WR-II showed smaller head and higher body compared with its original parents; the hybrid lineage and WR-II were diploid with 100 chromosomes; all the tested males and females of the hybrid lineage and WR-II had normal gonadal development and produced mature sperm and egg, respectively; the 5S rDNA of the hybrid lineage and WR-II inherited the characteristics of their parents and had some mutant bases or chimeric sequences, which revealed heredity, variability and heterozygosity in the hybrid progenies; finally, the 5S rDNA of the hybrid lineage could be highly inherited from the second generation (WR-$F_2$) to the next generations. This study will be beneficial for the establishment and application of hybrid lineages in genetic breeding.

## 1. Introduction

Fish genetic breeding research is an important work for aquaculture. To create new quality fish varieties or improve natural fish, many techniques have been applied in fish breeding research, including hybridization, selective breeding, gynogenesis, androgenesis, transgenosis, and gene editing (Wang et al., 2019). Many new types of fishes have been created through these breeding techniques, such as the formation of diploid, triploid and tetraploid hybrid fish using hybridization techniques (Liu, 2010; Song et al., 2012; Xu et al., 2015; Zhang et al., 2014); selective breeding of silver carp, rainbow trout, and yellow croaker has been reported (Gheyas et al., 2009; Kause et al., 2005; Ning et al., 2007); the tetraploid fish was improved using gynogenesis and androgenesis (Sun et al., 2007); and many commercial fish have been modified by transgenosis or gene editing (Hu et al., 2002; Rembold et al., 2006) because of the rapid development of transgenosis or gene editing techniques in fish. In China, by 2017, nearly half of the new national fish varieties created through breeding techniques were produced by hybridization (Wang et al., 2019). Therefore, the hybridization technique is the most common breeding technique in fish. Hybridization is defined as the mating of genetically differentiated individuals or groups, which is a common phenomenon in nature, whether in plants or in animals (Bartley et al., 2001; Zhang et al., 2014). Hybridization can combine whole genomes from parent species, which may lead to changes in both phenotype and genotype in the offspring (Liu, 2010; Liu et al., 2016). Thus, some advantageous traits can be produced by hybridization, and these traits are usually beneficial to production and breeding applications, such as body shape, growth and fertility (Huang et al., 2016).

White crucian carp (*Carassius cuvieri*, WCC) and red crucian carp

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(*Carassius auratus* red var., RCC) are diploid fish with 100 chromosomes, and both belong to Cypriniformes, Cyprinidae (genus *Carassius*). Previously, WCC and RCC were considered as the different varieties belonging to *Carassius auratus* (Liu et al., 2018a; Wang et al., 2015). Currently more and more studies indicated WCC and RCC should be classified into different species in the genus of *Carassius* (Luo et al., 2014; Rylková et al., 2010), and the Fishbase also described WCC and RCC as different species (www.fishbase.org). WCC and RCC have their own characteristics. WCC is characterized by silver body color, fast growth, weak stress resistance, and poor meat quality. RCC is characterized by red body color, strong stress resistance, perfect meat quality, and slow growth (Wang et al., 2015). Therefore, we designed a hybrid experiment using WCC and RCC as parents to produce a new type of fish with good meat quality, fast growth, and strong stress resistance. After many years of experimental research, we chose an optimal hybrid combination that used WCC as the female parent and RCC as the male parent to produce a new high quality fish (WR). In our previous reports, the morphology and genetic composition of WR-$F_1$ exhibited obvious hybrid traits, such as the gray body color being different from that of WCC and RCC, the growth rate was better than that of its parents, and there were many chimeric genes in the WR-$F_1$-$F_2$ (Liu et al., 2018a; Wang et al., 2015). WR-$F_1$ had higher protein content (17.7%) and lower moisture content (71.0%) compared with its parents (WCC: 14.8%, 75.6%; RCC: 17.0%, 75.5%); and the total flavor amino acids content of WR-$F_1$ (6.26%) was higher than that of RCC (6.07%), and was significantly higher that of WCC (5.29%) (Liu et al., 2017). Furthermore, we have successfully applied the gene knockout technique in WR-$F_1$ (Liu et al., 2018b).

In this study, we obtained a hybrid lineage (WR-$F_1$-$F_4$) by hybridization derived from WCC and RCC, which may ultimately evolve into a new species, and a new type of improved fish (WR-II) was obtained by back-crossing from the hybrid (WR-$F_1$) and the WCC. Our purpose is to research the genetic characteristics of the hybrid lineage and WR-II through the study of morphological traits, ploidy, fertility, and 5S rDNA; and provide a model system for the establishment and application of hybrid lineages in genetic breeding.

## 2. Materials and methods

### 2.1. Ethics statement

The guidelines established by the Administration of Affairs Concerning Animal Experimentation state that approval from the Science and Technology Bureau of China and the Department of Wildlife Administration is not necessary when the fish in question are neither rare nor near extinction (first- or second-class state protection level). Therefore, approval was not required for the experiments described in this manuscript. The fish were deeply anaesthetized with 100 mg/l MS-222 (Sigma-Aldrich, St. Louis, MO, USA) prior to dissection. Fish care and experimenters were certified under a professional training course for laboratory animal practitioners held by the Institute of Experimental Animals, Hunan Province, China.

### 2.2. Material and generation of hybrid populations

Individuals of WCC, RCC, WR-$F_1$-$F_4$ and WR-II were obtained from the Engineering Research Center of Polyploid Fish Breeding and Reproduction of the State Education Ministry, China, located at Hunan Normal University. During the reproductive season (from April to June) in 2014, mature parent fishes were selected. The WR-$F_1$ hybrids were formed by fertilization of haploid WCC eggs with haploid RCC sperm. Mature eggs were fertilized, and the embryos developed in the culture dishes at a water temperature of 20–22 °C. The hatched fry of WR-$F_1$ was transferred to a pond for further culture. During the reproductive season in 2015, 200 mature females and 200 mature males of WR-$F_1$ were chosen for self-breeding. The hatched fry of WR-$F_2$ were cultured under the same conditions as those for the WR-$F_1$ hybrids. During the reproductive season in 2016, The WR-$F_3$ hybrids were produced by the self-mating of WR-$F_2$. During the reproductive season in 2017, The WR-$F_4$ hybrids were produced by the self-mating of WR-$F_3$. The WR-II hybrids were formed by back-crossing WR-$F_1$ with WCC. The crossing procedures of the fish are shown in Fig. 1. Approximately 4000 embryos were taken at random from each of the WR-$F_1$-$F_4$ and WR-II hybrids for the examination of the fertilization rate (number of embryos at the stage of gastrula/total number of eggs taken for fertilization × 100%) and hatching rate (number of hatched fry/number of fertilized eggs × 100%).

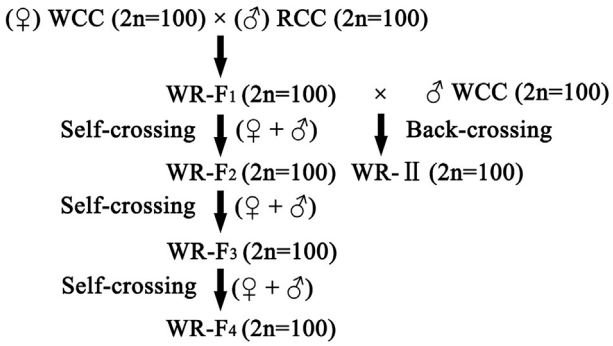


**Fig. 1.** Crossing procedure regarding the formation of the hybrid lineage and WR-II. WCC: white crucian carp; RCC: red crucian carp; WR-$F_1$: $F_1$ hybrid of female WCC and male RCC; WR-$F_2$: $F_2$ hybrid from the self-crossing of $F_1$; WR-$F_3$: $F_3$ hybrid from the self-crossing of $F_2$; WR-$F_4$: $F_4$ hybrid from the self-crossing of $F_3$; WR-II: hybrid of female WR-$F_1$ and male WCC. Self-crossing: crossing of brothers and sisters. Back-crossing: crossing of hybrid offspring and parents. 2n = 100: fish is diploid with 100 chromosomes.

### 2.3. Measurement of morphological traits and measurement of body weight for WR-II

We randomly selected 30 one-year-old fishes (total = 150) from WR-$F_1$-$F_4$ and WR-II for morphological examination. The countable traits include the numbers of scales in lateral lines, upper lateral lines and lower lateral lines, and the numbers of rays in dorsal fins, abdominal fins, and anal fins. The measurable traits include the average values of the whole length (WL), body length (BL), head length (HL), caudal peduncle length (CPL), body height (BH), head height (HH) and caudal peduncle height (CPH). Additionally, we translated the data of measurable traits into proportional data to more clearly show the morphological characteristics. In May of 2017, 200 WR-II were selected and reared in a 500-$m^2$ pond. After feeding for 3 months, body weights (BWs) of WR-II were measured for one year ($n$ = 50).

### 2.4. Examination of the ploidy level

The DNA content and the chromosome number were used to test the ploidy level of the hybrid offspring. The DNA content of the erythrocytes of WCC, RCC, WR-$F_1$-$F_4$ and WR-II were measured using a flow cytometer (Cell Counter Analyzer, Partec, Germany), and in each group 50 fish were sampled. Approximately 0.5–1 ml of blood from each fish was collected from the caudal vein using a syringe containing 100–150 units of sodium heparin. The blood samples were treated following the method described in the published paper (Liu et al., 2007). The DNA content of each sample was measured under identical conditions. The DNA contents of WCC and RCC were used as the controls. Finally, we used a $\chi^2$ test with Yate's correction to test for deviation in the ratios of the DNA content of the hybrid offspring to the sum of that from WCC and RCC from the expected ratio. The chromosome number was measured using kidney tissues from WR-$F_1$-$F_4$ and WR-II at ten months of age, respectively, and each sample included 10 fishes. The

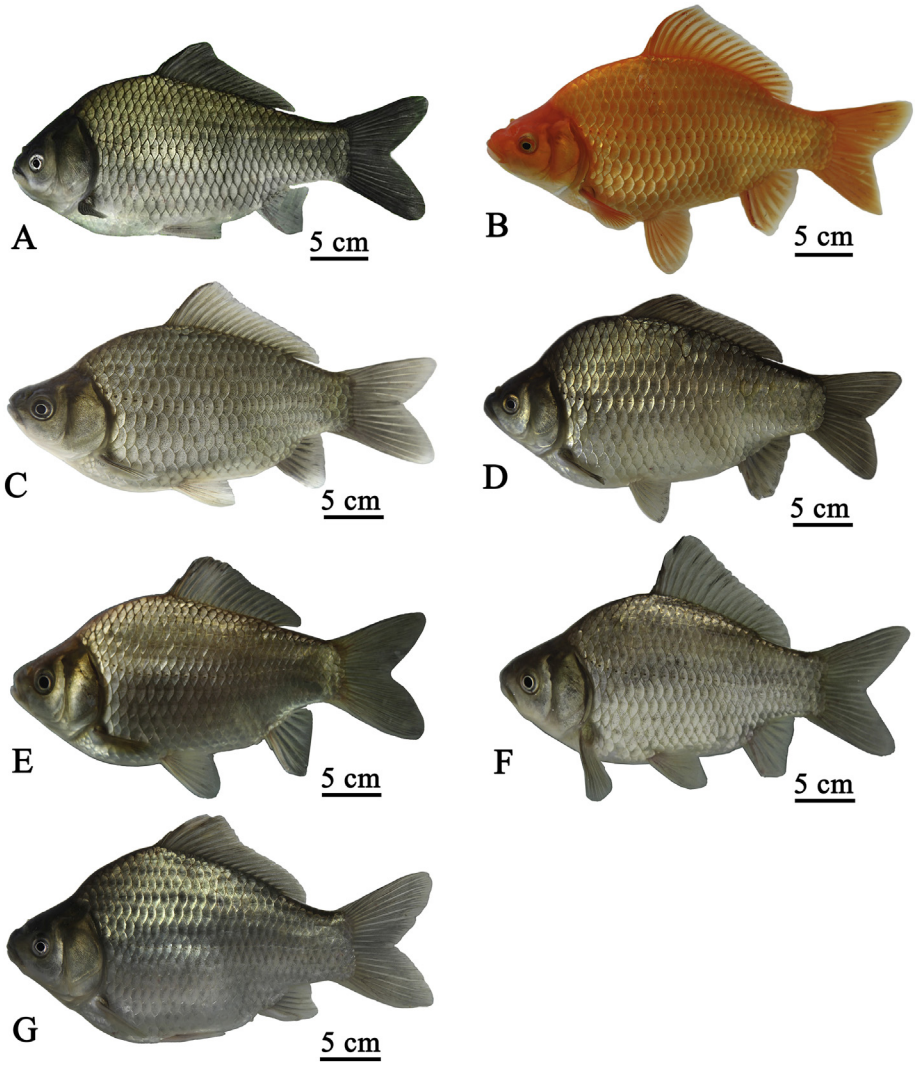


**Fig. 2.** The appearance of the original parents and their hybrids. (A) The appearance of white crucian carp (WCC), (B) the appearance of red crucian carp (RCC), (C) the appearance of the first generation of the hybrid linage (WR-$F_1$), (D) the appearance of the second generation of the hybrid lineage (WR-$F_2$), (E) the appearance of the third generation of the hybrid lineage (WR-$F_3$), (F) the appearance of the fourth generation of the hybrid lineage (WR-$F_4$), (G) the appearance of hybrid (WR-II) of female WR-F1 and male WCC. Bar = 5 cm.

**Table 1**
Morphological assessment of WCC, RCC, WR-$F_1$-$F_4$ and WR-II.

| Fish type | Lateral scales | Upper lateral scales | Lower lateral scales | Dorsal fins | Abdominal fins | Anal fins |
|---|---|---|---|---|---|---|
| WCC | 32–34 | 6–8 | 5–7 | III + 18–20 | 8–10 | III + 6–7 |
| RCC | 28–30 | 5–6 | 6–7 | III + 18–20 | 8–9 | III + 6–7 |
| WR-$F_1$ | 30–32 | 5–7 | 6–7 | III + 18–20 | 8–10 | III + 6–7 |
| WR-$F_2$ | 30–31 | 6–7 | 6–7 | III + 17–18 | 8–9 | III + 6–7 |
| WR-$F_3$ | 30–31 | 6–7 | 6–7 | III + 17–18 | 8–9 | III + 6–7 |
| WR-$F_4$ | 29–30 | 7–8 | 6–7 | III + 17–19 | 8–9 | III + 6–7 |
| WR-II | 30–32 | 6–7 | 6–7 | III + 18–21 | 8–9 | III + 6–7 |

preparations were made according to the method described by Liu et al. (Liu et al., 2007) with minor modifications. After culturing for two to three days at 20–22 °C, the samples were injected one to three times with concanavalin at a dose of 6–15 μg/g body weight at an interval of 12–24 h. Two to three hours prior to dissection, each sample was injected with colchicine at a dose of 4–6 μg/g body weight. The kidney tissue was ground in 0.9% NaCl and subjected to hypotonic treatment with 0.075 M KCl at 37 °C for 40–60 min and then fixed three times in 3:1 methanol-acetic acid. The cells were added dropwise to cold, wet slides and stained with 4% Giemsa for 40–60 min. The chromosome shape and numbers were analyzed under a light microscope. For each fish sample, 100 metaphase spreads (10 spreads per sample) were examined.

### 2.5. Gonadal structure and spermatozoa phenotype

WR-$F_1$-$F_4$ and WR-II individuals were randomly sampled at the age of ten months old for the examination of gonad development by histological sectioning, and in each group 10 fish were sampled. The gonads were fixed in Bouin's solution, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Gonadal structures were observed and photographed with a Pixera Pro 600ES digital camera (Nikon, Japan). The gonadal stages were classified in accordance with Liu's standard series for cyprinid fish (Liu, 1993).

At the age of one year, white semen was stripped from male individuals of WR-$F_1$-$F_4$ and WR-II. The semen was collected with a clean sucker and prefixed in 2.5% glutaraldehyde solution for the morphology examination. The samples were centrifuged at 2000 rpm for 1 min in an Eppendorf 5804R centrifuge (Eppendorf, Germany), then fixed in 4% glutaraldehyde solution overnight, and refixed in 1% osmic acid solution for 2 h. The samples were dehydrated in ethanol, dropped

**Table 2**
The ratios of measurable traits of WCC, RCC, WR-$F_1$-$F_4$ and WR-II.

| Fish type | WL/BL | BL/BH | BL/HL | HL/HH | CPL/CPH | HH/CPH |
|---|---|---|---|---|---|---|
| WCC | 1.24 ± 0.02 | 2.22 ± 0.15 | 3.70 ± 0.21 | 1.17 ± 0.06 | 0.81 ± 0.01 | 1.78 ± 0.09 |
| RCC | 1.23 ± 0.02 | 2.20 ± 0.16 | 3.72 ± 0.27 | 1.18 ± 0.04 | 0.79 ± 0.02 | 1.80 ± 0.07 |
| WR-$F_1$ | 1.25 ± 0.03 | 2.20 ± 0.15 | 3.70 ± 0.25 | 1.19 ± 0.06 | 0.82 ± 0.02 | 1.81 ± 0.12 |
| WR-$F_2$ | 1.24 ± 0.04 | 2.21 ± 0.13 | 3.73 ± 0.16 | 1.18 ± 0.07 | 0.79 ± 0.05 | 1.80 ± 0.03 |
| WR-$F_3$ | 1.24 ± 0.02 | 2.24 ± 0.12 | 3.73 ± 0.24 | 1.19 ± 0.02 | 0.81 ± 0.01 | 1.80 ± 0.06 |
| WR-$F_4$ | 1.24 ± 0.01 | 2.23 ± 0.09 | 3.75 ± 0.26 | 1.19 ± 0.07 | 0.81 ± 0.02 | 1.80 ± 0.04 |
| WR-II | 1.20 ± 0.01 | 1.97 ± 0.16[a] | 4.03 ± 0.34[a] | 1.03 ± 0.08 | 0.88 ± 0.11 | 1.23 ± 0.13[a] |

[a] Represents that the morphological characteristics of WR-II are significantly different from those of other fish types ($p < 0.05$).

**Table 3**
The mean body weight of WR-II (g).[a]

| Fish type | 3 months | 4 months | 6 months | 8 months | 10 months | 1 year |
|---|---|---|---|---|---|---|
| WR-II | 178 ± 7 | 227 ± 11 | 329 ± 18 | 419 ± 13 | 471 ± 23 | 556 ± 20 |

[a] Values are mean ± SE (g).

onto slides, desiccated, and subjected to atomized gilding before observation with a Hitachi X-650 scanning electron microscope (Nikon, Japan).

### 2.6. Genomic DNA extraction, PCR and sequencing

Total genomic DNA was extracted from the peripheral blood cells of WR-$F_1$-$F_4$ and WR-II using the Universal Genomic DNA Extraction kit (TaKaRa, Dalian, China). Two sets of primers (5S rDNA F: 5′-GCTATG CCCGATCTCGTCTGA-3′ and R: 5′-CAGGTTGGTATGGCCGTAAGC-3′) were designed by Primers 5 and synthesized to amplify the 5S rDNA from genomic DNA. The PCR reactions were performed in a volume of 25 μl with approximately 20 ng of genomic DNA, 1.5 mM $MgCl_2$, 250 μM of each dNTP, 0.4 μM of each primer, and 1.25 U of Taq polymerase (TaKaRa, Dalian, China). The thermal programme consisted of an initial denaturation step at 94 °C for 5 min followed by 35 cycles (94 °C for 35 s, 59 °C for 35 s and 72 °C for 35 s) and a final extension step at 72 °C for 5 min. The amplification products were separated on a 1.2% agarose gel using TBE buffer. After electrophoresis, the DNA fragments were purified using a gel extraction kit (Sangon, Shanghai, China) and ligated to the pMD18-T vector. The plasmids were transformed into *Escherichia coli* DH5α, and the DNA fragments inserted into the pMD18-T vector were sequenced using an automated DNA sequencer (ABI PRISM 3730, Applied Biosystems, CA, USA). The sequence homology and variation among the fragments amplified from WCC, RCC, WR-$F_1$-$F_4$ and WR-II were analyzed using BioEdit and Clustal W.

## 3. Results

### 3.1. Fertilization rates and hatching rates

The results of fertilization rates and hatching rates in the hybrid lineage and WR-II are shown in Table S1 in Supporting Information. In the crossing of WCC and RCC, back-crossing of WR-$F_1$ and WCC, and self-crossing of WR, we observed high fertilization rates (WR-$F_1$: 90.2%, WR-$F_2$: 91.3%, WR-$F_3$: 90.8%, WR-$F_4$: 90.7%, WR-II: 90.5%) and high hatching rates (WR-$F_1$: 81.5%, WR-$F_2$: 82.1%, WR-$F_3$: 82.7%, WR-$F_4$: 82.4%, WR-II: 80.3%) in the hybrid lineage and WR-II.

### 3.2. The morphological traits of the hybrid lineage and WR-II and the body weight of WR-II for one year

The phenotypes of WCC, RCC, WR-$F_1$, WR-$F_2$, WR-$F_3$, WR-$F_4$, and WR-II are illustrated in Fig. 2. The body color was all gray with no red in the hybrid progenies. The counts for lateral line scales, upper lateral line scales, lower lateral line scales, dorsal fin rays, anal fins rays, and abdominal fin rays in the parent species and hybrids are shown in Table 1. The ratios of WL/BL, BL/BH, BL/HL, HL/HH, CPL/CPH, and HH/CPH are indicated in Table 2. We observed no significant difference ($p > 0.05$) between the hybrid linage and its parents, i.e., WR-II and its parents, for all countable traits. Similarly, there was no significant difference ($p > 0.05$) between the hybrid lineage and its parents for all measurable traits. However, there was a significant difference ($p < 0.05$) in the ratios of BL/BH, BL/HL, and HH/CPH between WR-II and its parents, which indicated that WR-II had a smaller head and higher body. Mean BWs of WR-II are shown in Table 3. The mean body weight of one year old WR-II was 556 g.

### 3.3. DNA content and chromosome number of the hybrid lineage and WR-II

The DNA contents of WCC and RCC were used as the controls (Fig. 3 A and B, respectively). The results of the comparison of DNA content between hybrids and their parents are shown in Table S2 in Supporting Information. All the DNA content of the tested fish was equal ($p > 0.05$) to the sum of half of the contents for each of WCC and RCC, implying that the hybrid lineage and WR-II are diploid fish like their parents (Fig. 3C and D respectively). We tested 100 metaphase spreads of each type of hybrid fish, and the chromosome numbers of the hybrid lineage and WR-II are shown in Table S3 in Supporting Information. According to our results, > 94% of the tested metaphase spreads had 100 chromosomes, showing that they are diploid fish with 100 chromosomes (Fig. 3E and F respectively).

### 3.4. Fertility of the hybrid lineage and WR-II

At ten months old, the ovaries of the hybrid lineage and WR-II developed normally and were mainly composed of oocytes at stages II or III (Fig. 4A and B respectively). The testes of the hybrid lineage and WR-II were full of mature sperm (Fig. 4 C and D respectively). Furthermore, the white sperm or mature ova could be stripped from one-year-old hybrid fish. The sperm produced by the male hybrids had well developed heads and tails (Fig. 4 E and F respectively), showing that they had normal sperm morphology.

### 3.5. Nucleotide sequence of 5S rDNA in the hybrid lineage and WR-II

PCR amplification with 5S primers for the hybrid lineage and WR-II produced the same pattern with three bands (Fig. S1 in Supporting Information). We randomly selected six samples to clone 5S rDNA for

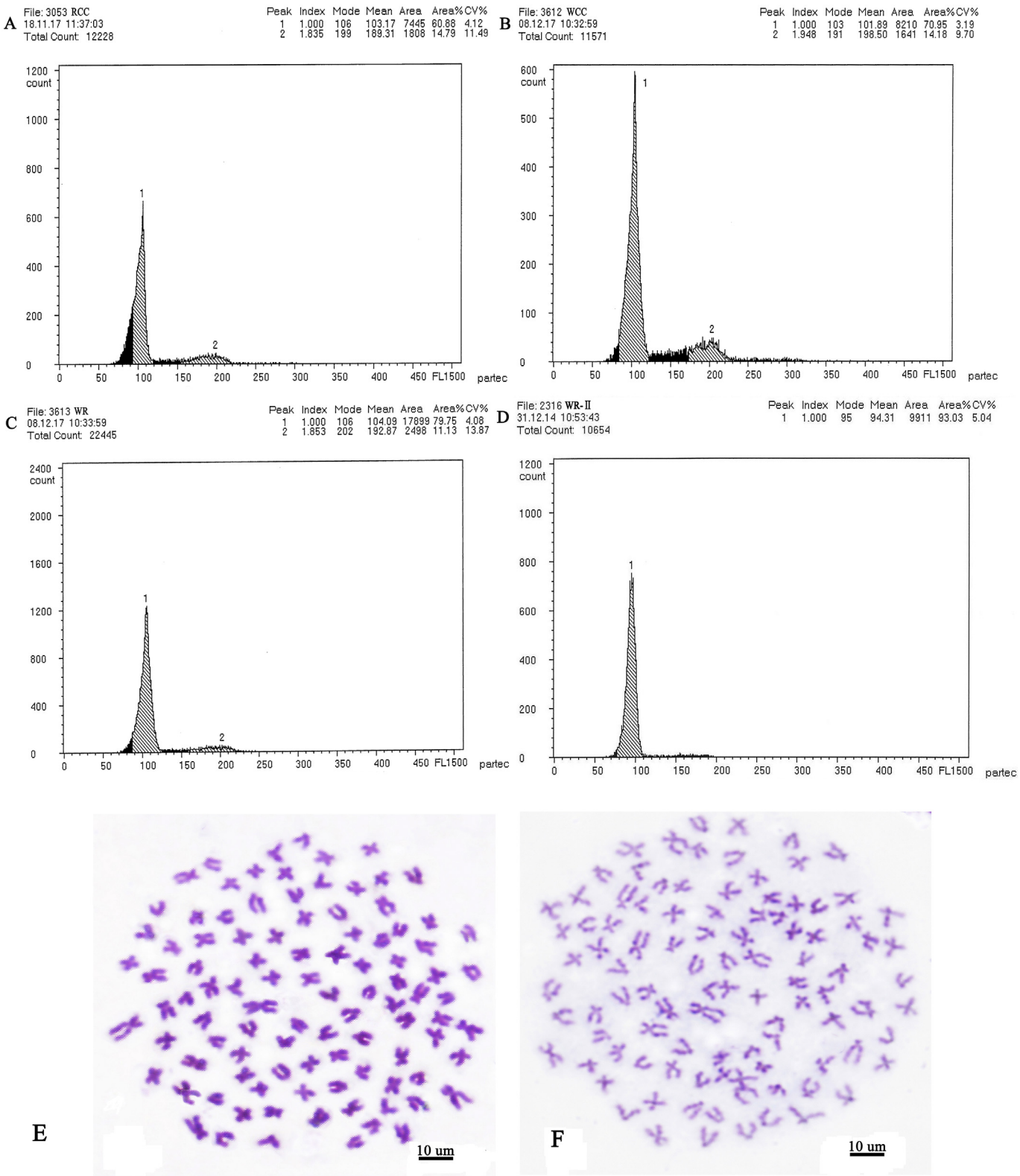


**Fig. 3.** Cytometric histograms of DNA fluorescence for WCC, RCC, WR (hybrid lineage) and WR-II, and the metaphase chromosome spreads of WR (hybrid lineage) and WR-II. (A) The mean DNA content of RCC (peak 1:103.17), (B) the mean DNA content of WCC (peak 1:101.89), (C) the mean DNA content of WR-$F_1$ (peak 1:104.09), (D) the mean DNA content of WR-II (peak 1:94.31), the metaphase chromosome spreads of WR (hybrid lineage) (2n = 100), and the metaphase chromosome spreads of WR-II (2n = 100). Bar = 10 μm.

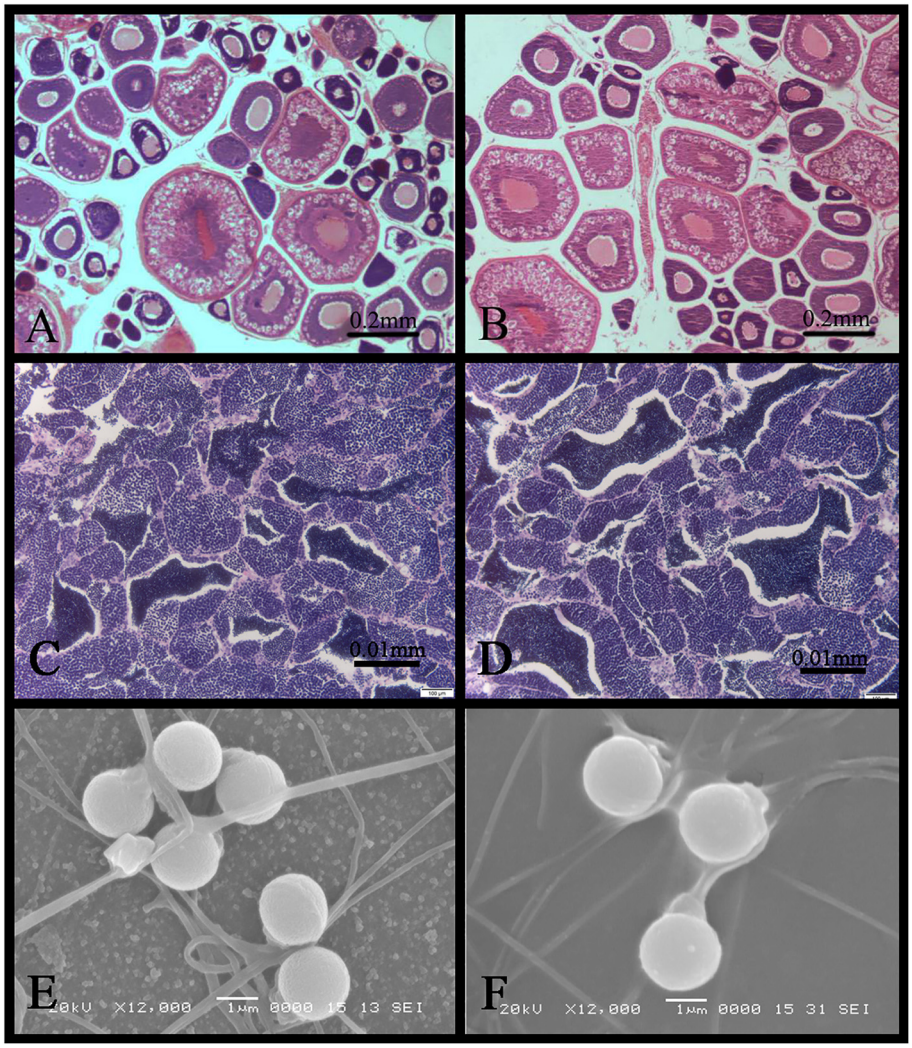


**Fig. 4.** Gonadal microstructure and the sperm structures of WR (hybrid lineage) and WR-II. (A) The histology of ovaries of WR (hybrid lineage), which showed oocytes at stages II or III, (B) the histology of ovaries of WR-II, which showed oocytes at stages II or III, Bar = 0.2 mm. (C) the histology of testis of WR (hybrid lineage), which had many mature spermatozoa and spermatids, (D) the histology of testis of WR-II, which had many mature spermatozoa and spermatids. Bar = 0.1 mm. (E) the structure of sperm of WR (hybrid lineage) under the scanning electron microscope, (F) the structure of sperm of WR-II under the scanning electron microscope. Bar = 1 μm.

each type of fish, and 30 clones were sequenced to examine the three bands (10 clones for each band and a total of 900 clones). The results of Sanger sequencing revealed that the sizes of the three fragments were 203 bp, 340 bp, and 471 bp, respectively. Using BLASTn, all the fragments were confirmed to be 5S rDNA repeat units, each comprising a 3′ end of the coding region (positions 100–120 in Fig. 5), a whole NTS region, and a large 5′ coding region of the adjacent unit (positions 1–99 in Fig. 5). Three fragments of 5S rDNA (designated class I: 203 bp, class II: 340 bp, and class III: 471 bp) were categorized into different NTS types (designated NTS-83, NTS-220, and NTS-351 for 83 bp, 220 bp, and 351 bp, respectively). All the internal control regions (including A box, internal element and C box) were identified in the coding regions (Fig. 5), and the TATA box control element, upstream from the next array, was examined within all the NTS sequences, which has been modified to TAAA (Figs. 6 and 7). The coding region sequence data of the hybrid lineage showed that the part of class I had many mutant bases compared with its parents rather than in class II and class III, and these mutant bases could be inherited in the next generations. In the WR-II, the coding region of 5S rDNA inherited the trait from WR-$F_1$ and with some mutant bases (Fig. 5). The NTS sequence data of the hybrid lineage showed that the NTS-83 (WR-$F_1$-$F_4$) had many mutant bases compared with its parents, and these mutant bases could be inherited by the next generations (Fig. 6A); the NTS-220 of WR-$F_1$ mostly inherited the genetic characteristics from its male parent RCC, but unlike WR-$F_1$, the NTS-220 of WR-$F_2$ mostly inherited the genetic characteristics from its female parent WCC and accompanied some mutant bases, and these genetic characteristics could be inherited in the WR-$F_3$ and WR-$F_4$ (Fig. 6B). The NTS-351 of WR-$F_1$ showed chimeric characteristics, including some specific-bases from WCC, some specific-bases from RCC, and a mutant base (position 7 in Fig. 7); similar to WR-$F_1$, the NTS-351 of WR-$F_2$ had chimeric characteristics accompanied by some genetic characteristics, and some genetic characteristics could be inherited by the WR-$F_3$ and WR-$F_4$. In the WR-II, the NTS-83 mostly inherited the female-specific (WR-$F_1$) bases and had a mutant base (position 4 in Fig. 6A); the NTS-220 mostly inherited its female-specific (WR-$F_1$) bases and had a mutant base (position 215 in Fig. 6B); however, the NTS-351 mostly inherited its male-specific (WCC) bases and had three mutant bases (position 300, 306, and 339 in Fig. 7).

## 4. Discussion

The hybridization breeding technique is widely used to produce offspring that exhibit hybrid vigor or positive heterosis in the genetic breeding of fish (Bartley et al., 2001). Many new types of fishes with dominant traits have been obtained by hybridization (Ren et al., 2016; Wang et al., 2017; Zhong et al., 2012). However, it is a challenge to establish a hybrid lineage because of the fertility of the progenies (Li et al., 2018). For instance, the hybrid offspring derived from female blunt snout bream (*Megalobrama amblycephala* Yih) and male Bleeker's yellow tail (*Xenocypris davidi* Bleeker) could not form a lineage because neither hybrids could produce mature eggs or sperm at 2 years of age (Hu et al., 2012). However, in our previous studies, a diploid hybrid

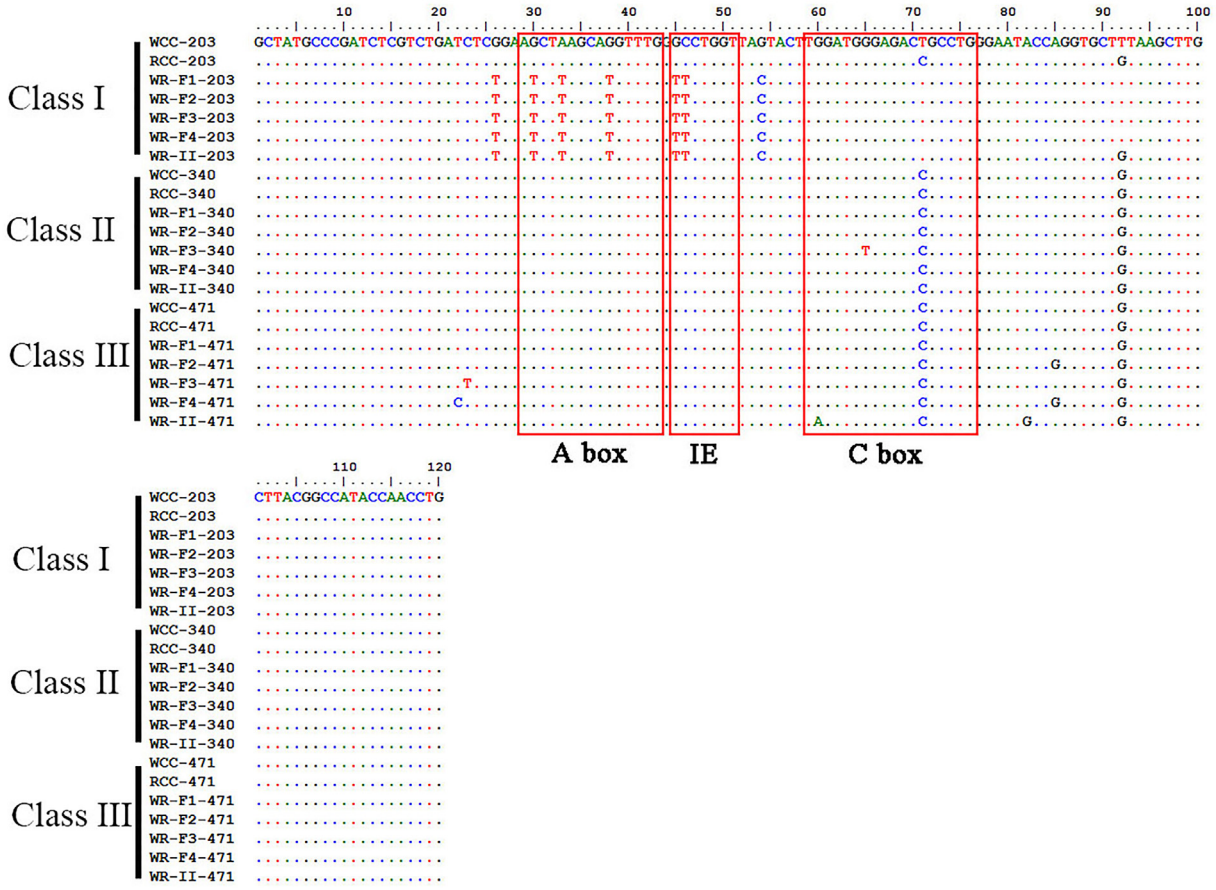


**Fig. 5.** Comparison of the 5S rDNA coding regions from WR-$F_1$-$F_4$ and WR-II. ICRs are included in the boxes.

lineage formed from the crossing of blunt snout bream and topmouth culter (*Culter alburnus*) (Xiao et al., 2014) and two types of tetraploid hybrid lineage derived from RCC and common carp (*Cyprinus carpio*) (Liu et al., 2001, 2007) and RCC and blunt snout bream (Qin et al., 2010) were established. In this study, we successfully established a fertile hybrid lineage by interspecific hybridization derived from WCC and RCC, and a new type of fish (WR-II) was obtained by back-crossing between WR-$F_1$ and WCC.

Usually, distant hybridization may be accompanied by the appearance of polyploidy, for example, when both hybrid females and males are able to produce diploid gametes, tetraploids can be produced (Liu et al., 2001, 2007), when some hybrid females can produce diploid eggs, the triploids can be produce by the crossing of these females with males of parental species (Liu, 2010). And, the first hybrid generation derived from the distant hybridization is usually infertility or reduced fertility because of the reproductive isolation between two different species. For example, the $F_1$ progeny resulting from hybridization between salmonid genera were sterile (Suzuki, 1973); the $F_1$ progeny derived from RCC and common carp had reduced fertility, and the $F_2$ hybrids were able to produce diploid eggs and diploid sperms that fertilized each other to form tetraploid fish in $F_3$ and eventually formed a tetraploid hybrid lineage ($F_3$-$F_{28}$) (Guo et al., 2006; Liu et al., 2016). Furthermore, diploid hybrids were difficultly surviving in distant hybridization, although diploid embryos were observed (He et al., 2012). In this study, the crossing of WCC and RCC belongs to interspecific hybridization because both of them belong to Cypriniformes, Cyprinidae (genus *Carassius*) (Luo et al., 2014; Rylková et al., 2010). According to our results, all the progenies in the lineage were diploid hybrid fish with 100 chromosomes, and there were no polyploidy fish (Fig. 3), and the males and females of the lineage had normal gonadal development, which could produce offspring with high fertilization rates and hatching rates (Table S1 in Supporting Information). Sexually fertile hybrid progeny provide a solid foundation for the formation of the hybrid lineage. On the other hand, although the genetic variability brought by interspecific hybridization is not as profound as that of supraspecific hybridization, there are still many dominant traits in interspecific hybridization. In our previous study, WR-$F_1$ showed a gray color that was different from its parents (silver white and red), and it had more rapid growth than that of its parents (Liu et al., 2018a,b; Wang et al., 2015). In this research, the WR-II showed a smaller head and higher body relative to those of its parents (Table 1); the mean body weight of one year old WR-II was 556 g (Table 3), and it was higher than WCC (395 g), RCC (246 g) and WR (350 g) which were measured in our previous study (Liu et al., 2018a). These phenotypes may be because back-crossing increases the genetic effect of its original female parent (WCC) so that it exhibits the characteristics of a smaller head, higher body and fast growth, similar to WCC. Finally, in some ways, the diploid hybrid lineage established by the crossing of parents with the same chromosomes provided evidence for the previous research findings, which stated that allotetraploid and allodiploid fish lineages could be produced when the number of maternal chromosomes was equal to that of paternal chromosomes in distant hybridization (Wang et al., 2019).

5S rDNA is a good marker for understanding the variability, organization, and evolution of fish species because of the special structure of the 5S rDNA multigene (Campo et al., 2009; Merlo et al., 2012). In this study, the formation of the hybrid lineage provided an ideal material for us to probe the genetic characteristics of 5S rDNA in the different hybrid generations. According to our results, we analyzed the 5S rDNA of the hybrid lineage and WR-II using three different views. For the first view, in the coding region, only class I had many mutant bases compared with its parents, and these mutant bases could be inherited by the

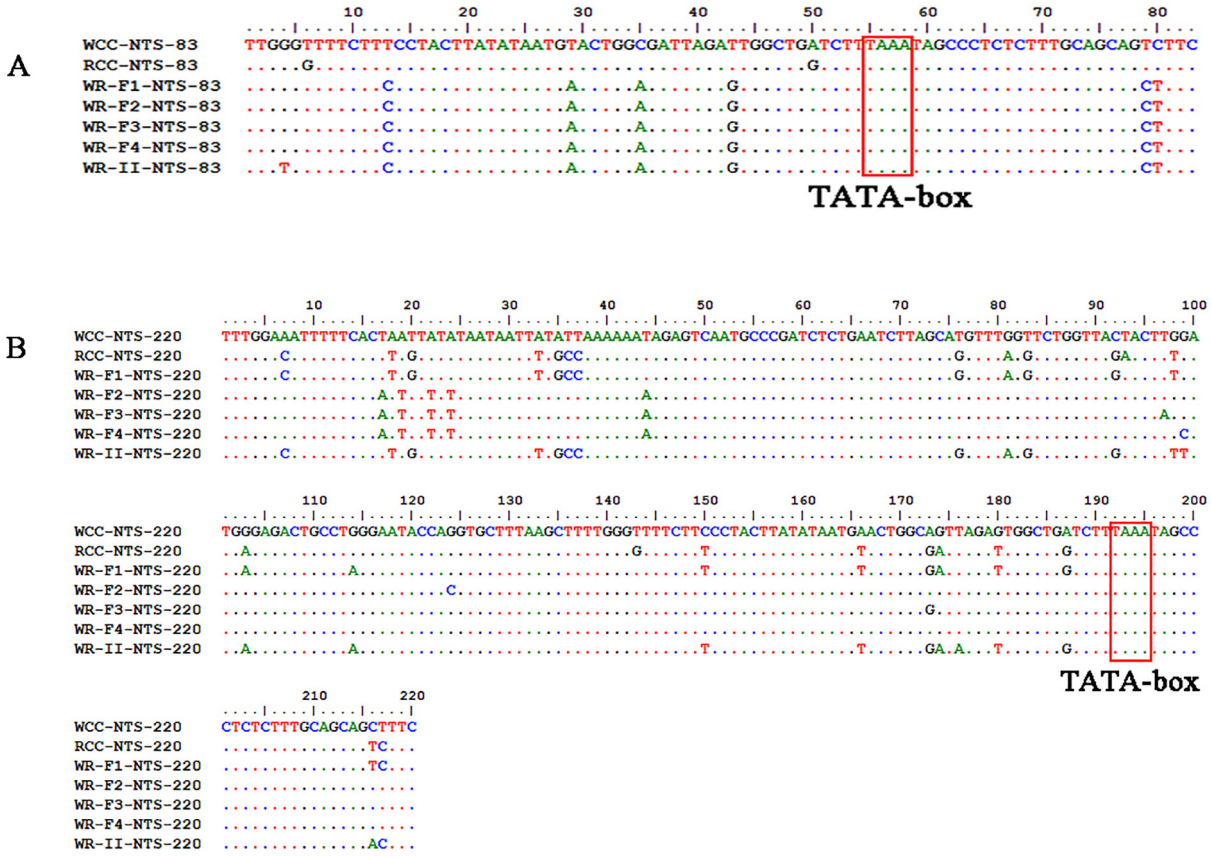


**Fig. 6.** Comparison of the NTS (83 and 220) sequences from WR-$F_1$-$F_4$ and WR-II. (A) Comparison of the NTS-83 sequences from WR-$F_1$-$F_4$ and WR-II, (B) comparison of the NTS-220 sequences from WR-$F_1$-$F_4$ and WR-II. The NTS upstream TATA-like sequences are included in the boxes.

next generations (Fig. 5); however, in the NTS region, the mutations occurred among in NTS-83, NTS-220, and NTS-351(Figs. 6 and 7), and some mutations were unstable between the different generations. This finding suggested that the coding region was more stable than the NTS region in the 5S rDNA, and class I was more stable than the other coding regions. Second, in the hybrid lineage, the sequence of NTS-220 and NTS-351 in the WR-$F_1$ could not be inherited by the WR-$F_2$, but the genetic characteristics of WR-$F_2$ could be inherited by the next generations (Fig. 6B and Fig. 7). This finding indicated that the first generation in the hybrid lineage was not stable, and the stability started in the second generation. However, in some other distant hybridization offspring, $F_2$ hybrids and those of further generation ($F_3$-$F_4$-…) have much larger variation than $F_1$ hybrids and many generations are needed to consolidate traits of interspecies hybrids in the row of generations, such as, hybrids striped bass x white bass (Avise and Van Den Avyle, 1984), hybrids channel catfish x blue catfish (Argue et al., 2014) and hybrids beluga x starlet (Havelka et al., 2017). That may be due to the relationship of the parents in these studies is farther apart than that in this study because WCC and RCC are very close species (Luo et al., 2014; Rylková et al., 2010). Finally, the 5S rDNA of the hybrid lineage and WR-II showed some sequences from its females or males, some sequences with mutant bases, and some sequences with chimeric characteristics. These results revealed heredity and variability in hybrid progeny at the molecular level. Furthermore, the results of the chimeric characteristics in the 5S rDNA were consistent with our previous research results, which showed 19.04% and 4.71% chimeric genes of orthologous genes from the livers of the WR-$F_1$ and WR-$F_2$, respectively (Liu et al., 2018a). However, what was the percent of chimeric genes in the WR-$F_3$, WR-$F_4$, WR-$F_5$…? In future research, we will investigate the chimeric genes in the hybrid lineage and WR-II and reveal the law of chimeric genes in the hybrid lineage.

In general, we established a hybrid lineage (WR-$F_1$-$F_4$) and produced a new type of fish (WR-II), and we showed the heredity and variability by analyzing the morphological traits, ploidy, fertility, and 5S rDNA. The formation of the lineage brought a new germplasm to produce some new types of fish (such as WR-II), and in further hybridization or breeding schemes, we will produce triploid fish with advantaged traits by the hybridization of WR-II and allotetraploid fish. Furthermore, the research provided evidence that interspecific hybridization was more likely to form a lineage than that in supraspecific hybridization.

## Author contributions

This study is conceived and designed by Q.F.L. and S.J.L. Most statistical analyses and write the manuscript by Q.F.L. Experimental work: Q.F.L., J.M.L., Q.L.L., Y.H.Q. Primers design: Q.F.L. and Q.L.L. Experimental materials collect: Q.F.L., Q.L.L., and J.M.L. Photographs collect: Q.F.L. Manuscript modify: S.J.L., B.C., M.T., C.Z., Q.B.Q. and J.W. All authors read and approve the final manuscript.

## Competing financial interests

The authors declare no competing financial interests.

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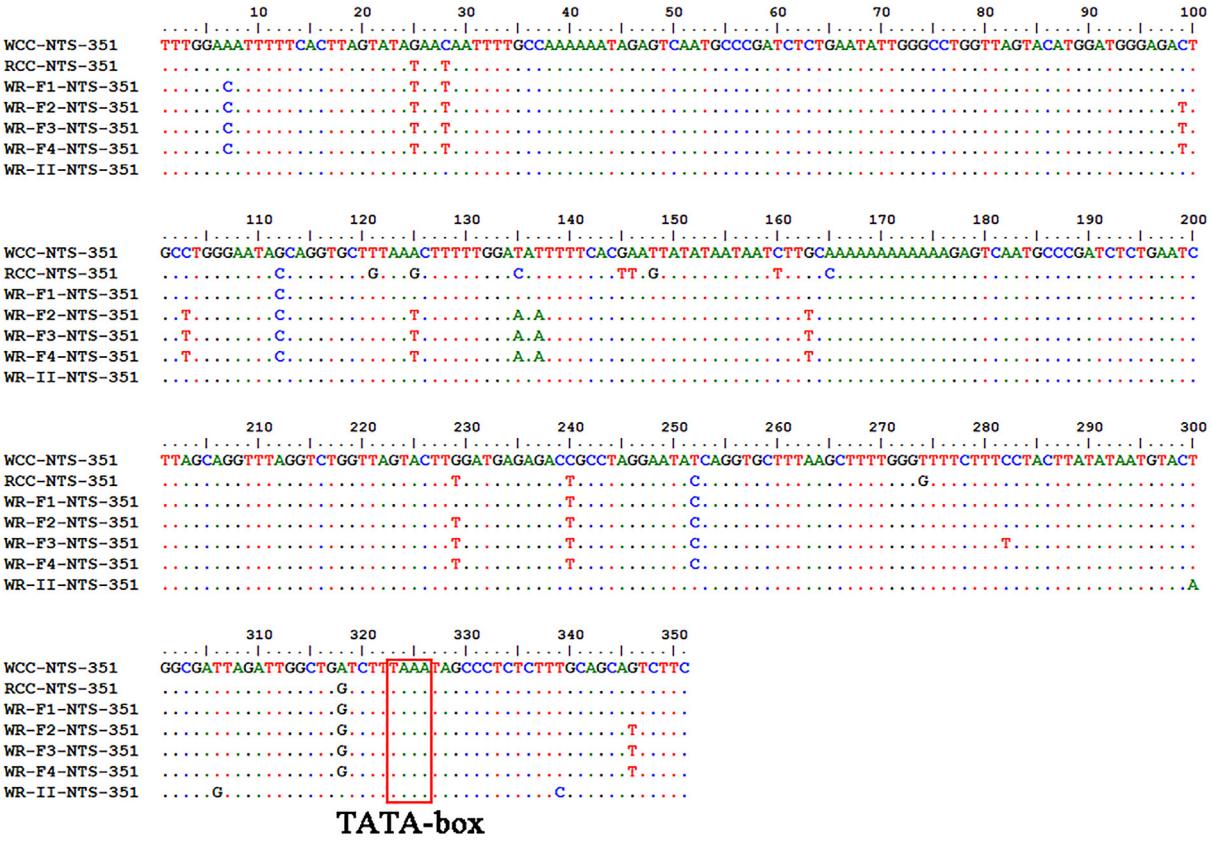


**Fig. 7.** Comparison of the NTS-351 sequences from WR-$F_1$-$F_4$ and WR-II. The NTS upstream TATA-like sequences are included in the boxes.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2019.02.056.

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